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Chapter

Gene Expression Profile of HDF in SMG Partially Overlaps with That in the NASA Twins Study

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Abstract

Microgravity research is an important field in biomedical sciences not only due to our interest in exploring and living in space, but also because of the insights it gives on earthbound health conditions. Using a human dermal fibroblast (HDF) cell line cultured in simulated microgravity (SMG) in combination with high throughput cDNA microarrays and quantitative Northern analysis, 271 differentially regulated genes were identified and 72% of these genes were also reported in the high throughput gene expression data of the recent National Aeronautics and Space Administration (NASA) Twins Study. The identification of the large number of overlapping microgravity sensitive genes between the skin fibroblast in microgravity and astronaut's peripheral blood mononuclear cells (PBMCs) indicated that microgravity alone, without space radiation, was able to elicit an adaptive response involving a set of about 200 genes. Further analysis of the overlapping genes with the same direction of regulation (86 genes) and opposite direction of regulation (108 genes) revealed important pathways and cellular processes in the microgravity adaptation responses.

Keywords: gene expression profiling, DNA microarray, northern blotting, rotating wall vessel (RWV), rotary cell culture system (RCCS), fibroblast, microgravity

1. Introduction

Humans have been traveling to space since 1961. During the close to 60-year period, hundreds of astronauts and cosmonauts have experienced microgravity as well as radiation in space. Exposure to microgravity environment has been shown to have potentially negative effects on human health. Some of the factors include cardiovascular deconditioning [1, 2], decline in immune response [3, 4], bone deterioration [5–7], and muscular atrophy [8–10].

Human immune response dysregulation has been shown during and after space flight [11]. Although acute onset life-threatening incidents directly caused by microgravity or spaceflight have not been reported, many conditions of health concern and symptoms related to immune dysfunction during orbital spaceflight have been described [12, 13]. Among the 70 tabulated clinical symptoms and medical conditions pertinent to immune dysfunction on board the International Space Station (ISS), skin rash and hypersensitivity account for 23 events; skin

infections, 6 events; cold sores (caused by herpes simplex virus 1 infection), 6 events. About 50% of the tabulated incidents are of or closely related to skin symptoms or abnormal skin conditions during the long duration space flights [12]. Skin is the essential outer cover that protects the internal tissues and organs from potential physical, chemical, and biological assaults of the environment. In addition to immune cells and keratinocytes, dermal fibroblasts also play an important immunomodulation role such as in antimicrobial defense [14]. Epidermal keratinocytes can sense the presence of pathogen invasion and other environmental stimuli such as the presence of UV light and foreign chemicals and produce cytokines, chemokines, and growth factors in response. Communication between keratinocytes and dermal fibroblasts through cytokines is fundamental in skin immunity. A recent report shows that in a 3D skin model-based study, just keratinocytes and fibroblasts alone embedded in a collagen matrix are able to activate CD4+ T cells in response to microbial invasion [15]. The dermal fibroblasts play an essential role in antimicrobial response by integrating signals among cells in the skin.

To date, much microgravity research work has been done in ground-based research using microgravity analogs. Due to cost and limits to the technology much less has been done directly in the space environment. The recent NASA Twins Study is a tremendously important study because it is the first study that uses an integrated approach to study human adaptation to a space environment by documenting the molecular, physiological and cognitive effects during long term spaceflight [16]. The study also highlights the need for further study on important aspects such as vascular changes and immunological stress associated with the weightlessness of space flight [16].

Sudden gravity change has altered gene expressions from many cell types [17–19]. High-throughput gene expression analysis have great potential for application to research involving changes in environmental conditions [20]. Various high throughput studies such as cDNA microarrays and transcriptome RNA sequencing have been increasingly used to assess the mRNA levels in microgravity research [16, 17]. This is an effective approach because the control of mRNA abundance of genes is efficiently adapted by cells through controlling transcription (especially transcription initiation), nuclear pre-mRNA processing, mRNA transport, mRNA stability, etc. The cellular abundance of mRNAs is critical to gene function and protein production which are intriguingly fine-tuned by non-coding regulatory RNAs such as miRNAs. There have been many gene expression studies done on various cell lines grown both in space and using ground-based microgravity analogs. Many of these studies have yielded valuable data, but correlation of gene expression data between studies has been relatively low [17, 18].

To further understand the cellular and molecular mechanisms by which space flight alters skin immune defense activities such as those analyzed from the ISS [12], the effects of microgravity on various human skin cell lines need to be studied to identify the genes whose functions are altered by microgravity. In our previous study, we found expression changes in certain genes (such as HLA-G and IL-1β among many others) in response to simulated microgravity [21]. The current report is on the gene expression profile of HDF in response to SMG. Interestingly, a substantial overlap in gene expression profiles between the HDF under SMG and that from the human blood cells of the NASA Twins Study, especially the peripheral blood mononuclear cells (PBMCs) from inflight in the ISS. The comparative analysis yielded 194 differently expressed genes in both studies, of which 86 genes were regulated in the same direction (trend) while 108 genes were regulated oppositely. The significance of these findings was discussed.

2. Materials and methods

2.1 Simulated microgravity and cell culture

The HDF cell line, AG 1522, was generously provided by Dr. Honglu Wu of NASA. The HDF cell line displayed regular monolayer spindle shaped growth in conventional 2-D cell culture flasks. When HDF cells were subjected to SMG treatment, in a 3-D culture environment, they formed spherical aggregates. Ground based simulated microgravity was achieved using the 50 ml high aspect ratio vessels (HARVs) or rotating wall vessels (RWVs) of a rotary cell culture system (RCCS-4D) bioreactors from Synthecon, Inc. Cell viability and cell concentration were determined by Vi-Cell 1.01 cell counter of Beckman Coulter. For the three parallel experiments of 5 day modeled microgravity exposures, a density of 2.0×10^6 cells/ml with viability of 95.5% of the HDF AG1522 cells were cultured in RWVs at 20 rpm rotary setting to achieve the constant free-fall experience for cell aggregates. At the end of the five-day microgravity exposure period, the content of the bioreactor vessels was poured out into a 50 ml sterile centrifuge tube to collect cell pellet and 5 ml of the cell suspension from the bioreactor vessels were transferred to T75 flasks for morphological observation. Non-exposed stationary normal gravity control AG1522 cells were cultured in tissue culture flasks with vented caps (TPP Techno Plastic) in the same incubator at 37°C, 5% CO_2.

2.2 Total RNA isolation and DNA microarray hybridization

HDF cells cultured in three SMG bioreactor vessels and control flasks were removed at the end of the five-day SMG exposure period, washed with phosphate buffered saline three times and lysed in Guanidinium Isothiocyanate Buffer. The cell lysates were stored at −80°C prior to ultracentrifugation for total RNA isolation [22, 23]. Total cellular RNA was labeled using the Agilent Low RNA Input Fluorescent Linear Amplification Kit following manufacturer's protocols [24]. The fluorescently labeled cRNA probes were further purified and hybridized to Agilent 22 K Human Microarray V2 according to the specified procedures within the kit.

2.3 Microarray scanning, feature extraction and functional grouping

The microarrays were scanned using a ScanArray microarray scanner (Perkin-Elmer). The images generated from the scanning were imported into GenePix 6.0 (Molecular Devices, Sunnyvale, CA) for alignment and initial quantitation. The Gene Pix Results (GPR) files were then uploaded to CARMAweb [25] for normalization and statistical analysis. Background was subtracted and then each array was normalized using loess normalization within the array. A paired moderated T-Test was applied with Benjamini-Hochberg correction to control the false discovery rate [26]. Cut-offs were set at a P-value ≤ 0.01 and fold change of ≥ 1.5.

2.4 Northern blotting and quantitative gene expression analysis

Some of the significantly regulated microgravity sensitive genes identified from the DNA microarray analysis were further verified using Northern blotting. Briefly, 10 ug total RNA was loaded per lane on a 1% formaldehyde agarose gel for electrophoresis separation of RNA species. RNA Ladders from Fermentas Life Sciences were used as RNA size markers. The gel-separated RNAs were capillary transferred onto a nylon membrane which was subjected to a hybridization procedure using

chemiluminescent (Pierce Biotechnology) labeled cDNA probe fragments. The blot was sequentially hybridized and striped and hybridized again with 12 different probe fragments. The cDNA probe fragments were generated from reverse transcription polymerase chain reaction (RT-PCR) using total cellular RNA as the templates. RT-PCRs were carried out using the Reverse Transcription System of Promega and the BioLine Red Polymerase PCR kit. Northern blot quantitation and association with the cDNA array results were done as described previously [21, 23].

2.5 Comparative analysis with the data from the NASA twins study

The NASA Twin gene expression data was identified from Suppemental Table 2 in the NASA Twins Study [16]. Prior to further bioinformatics analysis, the high throughput gene expression data from transcriptome RNA sequencing analysis for the inflight, first half and in-flight, second half was extracted from the NASA Twin study [16]. This data was then compared to the gene expression data from the current study with HDF in SMG. Mainly the transcriptome RNA sequencing data of the PBMC RNA from NASA Twins Study was compared with the HDF SMG data here, since it offered the most abundant overlapping genes. The PBMC data was extracted from the Excel spreadsheets and combined with the HDF data for further comparative analysis using similar previously published method [17].

2.6 Pathway and gene ontology analysis

To determine the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways [27, 28] and Gene Ontology, the genes that were determined to be differentially regulated were uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) [29]. The pathway and gene ontology information were used to build the tables. The process was done for both the HDF SMG data alone, and for its comparative analysis with the data from the NASA Twins Study. Prior to further bioinformatics analysis, the gene expression data for the inflight, first half and in-flight, second half was extracted from Supplemental Table 2 of the NASA Twins study [16]. This data was then compared to the gene expression data from the current study with HDF in SMG.

2.7 Constructing heat map and Venn diagram

The heat map was generated using Genesis 1.8.1 [30]. The Venn diagram was generated using the Excel plugin Array File Maker 4.0 [31]

3. Results

3.1 Gene expression profiling and the identification of microgravity sensitive genes from SMG treated HDF

HDF cells from each of the three SMG bioreactors and normal gravity controls were removed after 5-day SMG exposure for RNA extraction and microarray experiments. After normalization and statistical analysis (student *t* test), the gene expression data were used to identify the initial set of significantly differentially regulated genes at the statistically significance level of $P \leq 0.01$ and cut off point of ≥ 1.5 fold up or down regulation. The volcano plot (**Figure 1**) shows the overall profile of the gene expression data from the three sets of parallel SMG experiments. Each dot on the volcano plot represents a gene selected in the initial set of differentially

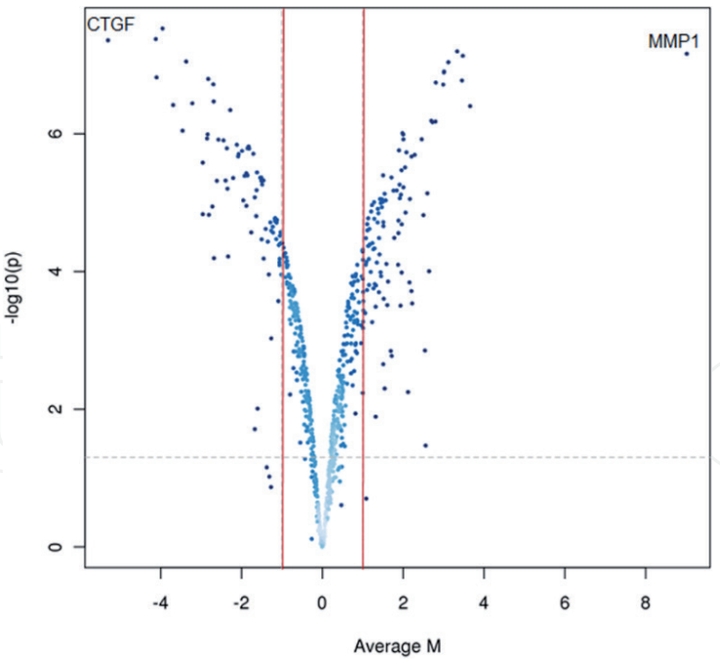

Figure 1.
Volcano plot scattering the average M values (x axis) against the corrected p values (y axis) created using CARMAweb tool. Dots to the left of the red line and above the dashed line have a p-value of 0.01 or less and are down-regulated by at least 1.5 fold. The dots to the right of the red line and above the dashed lines have a p-value of 0.01 or less and are up-regulated by at least 1.5 fold.

expressed ones if the dot is to the left (down) or right (up) of the red lines. The genes found outside of the vertical parallel red lines correspond to the genes differentially expressed by 1.5 fold or greater in the SMG. A total of 271 genes were identified from these three SMG experiments on HDF to be the initial set of significant genes that were used for further analysis as follows. The two most notable differentially regulated genes by the SMG evident on the plot were the matrix metallopeptidase 1 (MMP1) and connective tissue growth factor (CTGF) genes (**Figure 1**).

The significantly ($\geq$ 1.5 fold change, $P \leq 0.01$) differentially expressed genes under simulated microgravity were further analyzed using Heatmap and Venn diagram to visually display the directions and centralization of gene expression. A high level of consistency of both the Heatmap and Venn diagram results were found among the microarray data from the three SMG bioreactors (**Figure 2**). The Heatmap indicated that the expression levels of the three replicates were similar, with very minor variations in magnitude of expression. The Venn diagram further showed that there was a complete match among the three replicate microarrays from the three SMG bioreactor RNA samples. Thus, there was a very high level of consistence among data from the three SMG bioreactors. Among the 271 microgravity sensitive genes that differentially regulated by 1.5 fold or greater with a P value of $\leq$0.01, 129 were down-regulated and 142 were upregulated (**Figure 2B**).

3.2 KEGG pathways of the microgravity sensitive genes from HDF

The identified 271 SMG sensitive genes were subjected to further bioinformatics analysis using the DAVID v6.8, which uses a modified Fisher Exact P-value for gene enrichment analysis and statistically determines the over-representation of functional gene categories in a gene list. P values equal to or smaller than 0.05 are considered significantly enriched [29]. Through the DAVID analysis, 16 statistically significant ($p \leq 0.05$) KEGG Pathways from the SMG gene list were identified and genes in all the 23 KEGG pathways had >2 fold enrichment (**Table 1**).

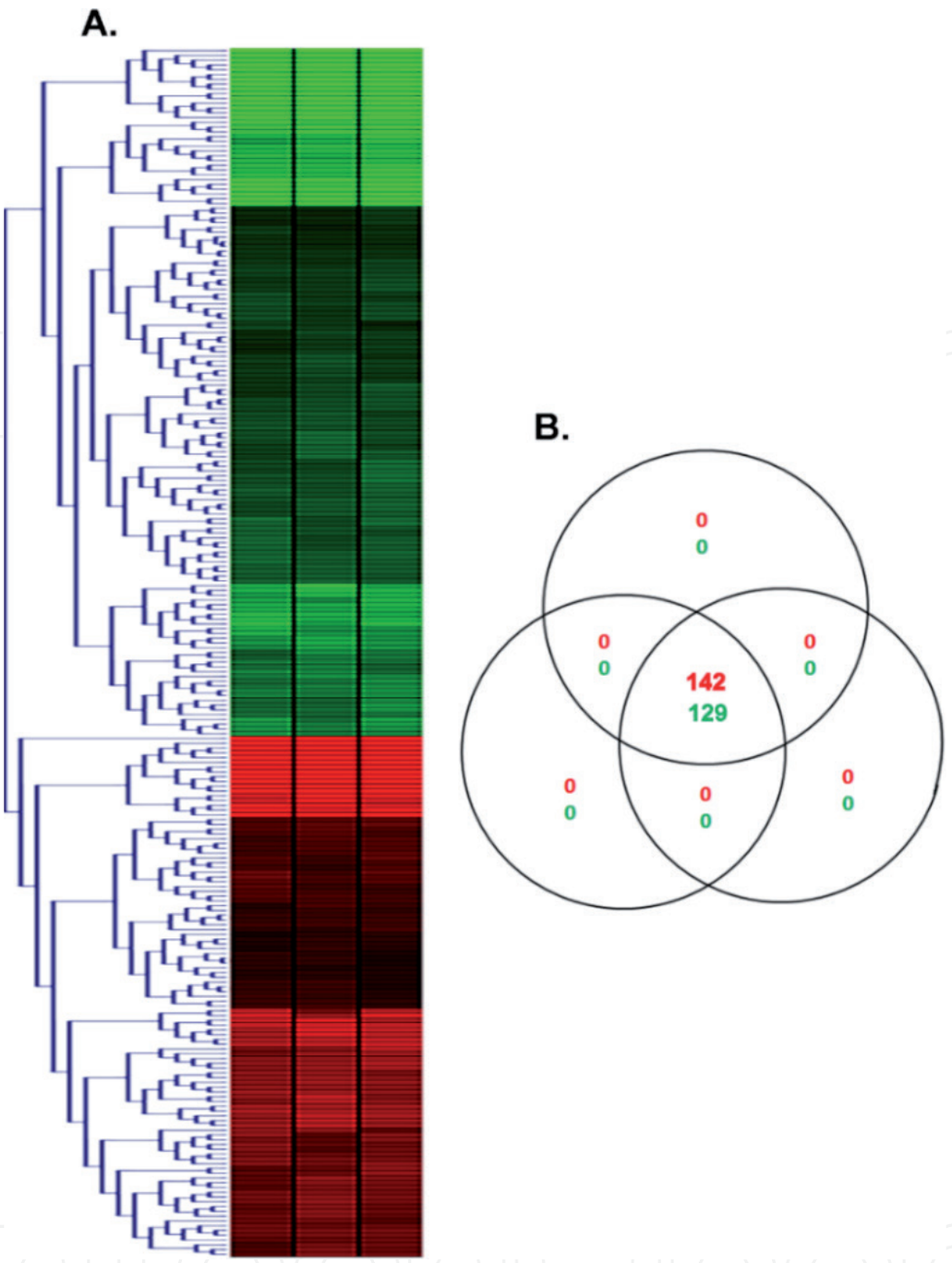

Figure 2.
Heatmap and Venn diagram comparing the expression levels among the HDF cells cultured in three separate bioreactors. (A) The Heatmap shows the expression levels were very similar among the three replicate experiments. Green represents the down-regulated genes and red represents the up-regulated genes. (B) Venn diagram comparing the centralization level of the differentially regulated genes among each of the three replicates. The red represents the up-regulated genes and the green represents the down-regulated genes.

The Ribosome KEGG Pathway included 27 of the SMG sensitive genes; 26 of these genes were down-regulated between 1.5 and 2 fold; only MRPS6 was upregulated (4.5 fold). Interestingly, a number of ribosomal protein genes have also been found to be down-regulated in other studies including our previous study on keratinocytes [21] as well as the NASA Twins Study [16]. The mineral absorption pathway had 10 genes which were all significantly up-regulated in SMG. ATP1A1 and ATP1B3 were both up-regulated by over 2.8 fold. The ferritin genes FTH1 and FTL were up-regulated by 6.6 and 1.6 fold, respectively. Ferritin genes have also been found to be up-regulated as a result of exposure to simulated microgravity in other studies [32]. The metallothionein genes were up-regulated from 7.5 to close to

Pathways	P value	Gene name	FE
hsa03010: Ribosome	1.25×10^{-16}	Ribosomal Protein L7(RPL7), L9 (RPL9), L10A (RPL10A), L11 (RPL11), L12 (RPL12), L18A (RPL18), L27 (RPL27), L27A (RPL27A), L31 (RPL30), L32 (RPL32), L34 (RPL34), L35 (RPL35), L36 (RPL36), L39 (RPL39), S2 (RPS2), S3 (RPS3), S3A (RPS3A), S7 (RPS7), S10 (RPS10), S15A (RPS15A), S17 (RPS17), S18 (RPS18), S19 (RPS19), S29 (RPS29), S23 (RPS23), S24 (RPS24), Mitochondrial Ribosomal ProteinS6 (MRPS6)	7.99
hsa04978: Mineral absorption	9.38×10^{-7}	ATPase Na+/K+ Transporting Subunit Alpha 1 (ATP1A1), ATPase Na+/K+ Transporting Subunit Beta 3 (ATP1B3), Metallothionein 1A (MT1A), 1B (MT1B), 1G MT1G), 1H (MT1H), 1X (MT1X), 2A (MT2A), Ferritin Heavy Chain 1(FTH1), Ferritin Light Chain (FTL)	9.14
hsa04510: Focal adhesion	1.61×10^{-4}	Actin Beta (ACTB), Actin Gamma 1(ACTG1), Caveolin 1 (CAV1), Collagen Type 3 Alpha 1 Chain (COL3A1), Type I Alpha 1 Chain (COL1A1), Type 1 Alpha 2 Chain (COL1A2), Type 6 Alpha 3 Chain (COL6A3) Actinin Alpha 1, Myosin Light Chain 9 (MYL9), 12A (MYL12A), 12B (MYL12B), Calpain 2 (CAPN2), Integrin Beta 1(ITGB1), Filamin A (FLNA),, Vascular endothelial growth factor B (VEGFB), Fibronectin 1(FN1)	3.12
hsa05205: Proteoglycans in cancer	1.30×10^{-3}	ACTB, ACTG1, Cathepsin L (CTSL), Human leukocyte antigen A (HLA-A), B (HLA-B), C (HLA-C), G (HLA-G), Hepatocyte Growth Factor (HGS), Tubulin Beta 6 Class V (TUBB6), Tubulin alpha 1 B (TUBA1B), ITGB1,	2.82
hsa04612: Antigen processing and presentation	2.60×10^{-3}	Beta-2-Microglobulin (B2M), CTSL, HLA-A, HLA-B, HLA-C, HLA-G, Protein Family A (Hsp70) Member 1A Shock Protein (HSPA1A), Protein Family A (Hsp70) Member 8A (HSPA8)	4.23
hsa05012: Parkinson's disease	2.61×10^{-3}	Peptidylprolyl Isomerase F (PPIF), NADH–ubiquinone oxidoreductase chain 1 (ND1), NADH–ubiquinone oxidoreductase chain 3 (ND3) ATP synthase F1 subunit epsilon (ATP5E), ATP Synthase Peripheral Stalk Subunit OSCP (ATP50), NADH:Ubiquinone Oxidoreductase Subunit B4 (NDUFB4), Ubiquinol-Cytochrome C Reductase, Complex III Subunit XI (UQCR11), Cyclooxygenase 1 (COX1) and 2 (COX2), Ubiquitin C-Terminal Hydrolase L1 (UCHL1), Ubiquitin B (UBB)	3.12
hsa05416: Viral myocarditis	2.67×10^{-3}	ACTB, ACTG1, CAV1, HLA-A, HLA-C, HLA-B, HLA-G	4.94
hsa04145: Phagosome	3.88×10^{-3}	ACTB, ACTG1, CTSL, HLA-A, HGS, TUBB6, HLA-C, HLA-B, TUBA1B, ITGB1, HLA-G	2.95
hsa05130: Pathogenic *Escherichia coli* infection	8.15×10^{-3}	ACTB, ACTG1, ARPC2, TUBB6, TUBA1B, ITGB1	4.73
hsa04141: Protein processing in endoplasmic reticulum	8.86×10^{-3}	Heat Shock Protein 90 Beta Family Member 1 (HSP90B1), Valosin Containing Protein (VCP), Defender Against Cell Death 1 (DAD1), DnaJ homolog subfamily A member 1 (DNAJA1), Protein Disulfide Isomerase Family A Member 4 (PDIA4) and 6 (PDIA6), HSPA1A, Heat Shock Protein Family A (Hsp70) Member 5 (HSPA5), CAPN2, HSPA8, Signal Sequence Receptor Subunit 1 (SSR1)	2.62

Pathways	P value	Gene name	FE
hsa00190: Oxidative phosphorylation	1.69×10^{-2}	ND1, ATP5E, NDUFB4, UQCR11, COX2, COX1, ND3, ATP5O, PPA1	2.72
hsa04530: Tight junction	2.03×10^{-2}	ACTB, ACTG1, ACTN1, MYL12B, MYL12A, member RAS oncogene family(RAB13), MYL9	3.24
hsa04512: ECM-receptor interaction	2.03×10^{-2}	CD44, COL3A1, COL6A3, COL1A2, COL1A1, ITGB1, FN1	3.24
hsa04974: Protein digestion and absorption	2.13×10^{-2}	HSP90B1, VCP, DAD1, DNAJA1, PDIA6, HSPA1A, HSPA5, PDIA4, CAPN2, HSPA8, SSR1	3.2
hsa04670: Leukocyte transendothelial migration	2.34×10^{-2}	ACTB, ACTG1, ACTN1, MYL12B, MYL12A, ITGB1, MYL9, THY1	2.8
hsa04260: Cardiac muscle contraction	3.75×10^{-2}	UQCR11, ATP1B3, COX2, COX1, ATP1A1, tropomyosin 2 (beta) (TPM2)	3.22
hsa04611: Platelet activation	4.17×10^{-2}	ACTB, ACTG1, COL3A1, COL1A2, MYL12B, COL1A1, MYL12A, ITGB1	2.48
hsa05100: Bacterial invasion of epithelial cells	4.32×10^{-2}	ACTB, ACTG1, CAV1, ARPC2, ITGB1, FN1	3.09
hsa05332: Graft-versus-host disease	4.71×10^{-2}	HLA-A, HLA-C, HLA-B, HLA-G	4.88
hsa05010: Alzheimer's disease	5.59×10^{-2}	tumor necrosis factor receptor superfamily member 1A(TNFRSF1A), ATP5E, NDUFB4, UQCR11, COX2, COX1, ATP5O, CAPN2, Calmodulin 2 (CALM2)	2.16
hsa05330: Allograft rejection	6.25×10^{-2}	HLA-A, HLA-C, HLA-B, HLA-G	4.35
hsa05131: Shigellosis	7.30×10^{-2}	ACTB, ACTG1, CD44, ARPC2, ITGB1	3.14
hsa04940:Type I diabetes mellitus	8.45×10^{-2}	HLA-A, HLA-C, HLA-B, HLA-G	3.83
hsa04918:Thyroid hormone synthesis	9.43×10^{-2}	HSP90B1, ATP1B3, ATP1A1, HSPA5, PDIA4	2.87

Red indicates that the genes were up-regulated in the data and green indicates the genes were down-regulated in the data. The significance of gene enrichment is evaluated chiefly by the p value with a modified Fisher's exact test. Fold enrichment (FE) is a measure of the magnitude of gene enrichment. P value ≤0.05 and FE ≥1.5 were considered significant and interesting.

Table 1.
KEGG pathway analysis of the 271 differential regulated genes from the HDF cells exposed to 5 days SMG.

12 fold. In a previous study on human keratinocytes in SMG, metallothioneins were also up-regulated [21]. Metallothionein isoforms MT1 and MT2 have been identified as gravity sensitive genes [17, 18, 21, 33–35]. Focal adhesion pathway genes were found to be generally down-regulated by more than 2 fold in the present study. The only exceptions were ITGB1 and VEGFB which were up-regulated by 1.5 and 1.8 fold, respectively. There were 14 genes in the proteoglycans in cancer category, with 9 genes being up-regulated and 4 down-regulated; these included several major gravity sensitive genes identified previously, CAV1, FN1, DCN, and CD44 [17].
In the antigen processing and presentation pathway, the 8 genes represented were all up-regulated. These include the HLA genes, the closely related B2M, as well as CTSL, HSPA1A and HSPA8. In a previous study, HLA-G is also up-regulated in the SMG treated human keratinocytes [21]. In normal gravity environment, HLA-G has been shown to have direct inhibitory effect on T, APC, and NK cell functions and induces suppressor T-cells [36]. It has been found to be present in neurological

disorders [37]. HLA-G is considered a stress inducible gene [38]. HLA-G also plays a role in tumor-driven immune escape mechanism of cancer cells during the later phase in host and tumor cell interactions. When HLA-G is expressed, it can result in an immune suppressive function [38–40]. HLA-G expression has been found to correlate with low frequency of rejection in some forms of organ transplants [39]. Heat shock proteins HSPA1A and HSPA8 have been identified as being gravity sensitive in several studies. In some of these studies, HSPA1A has been shown to be up-regulated [21, 32, 41]. They were also shown to be down-regulated [42].

3.3 Validation of microarray results through northern blotting analysis

The quality of a gene list from a high throughput study is essential for a successful functional analysis in DAVID [29]. The high throughput microarray data of HDF in SMG presented above was further validated by performing Northern blot analysis.

Northern blotting measures the abundance as well as the size of the RNA of interest [21]. In agreement with the microarray data, the Northern results showed

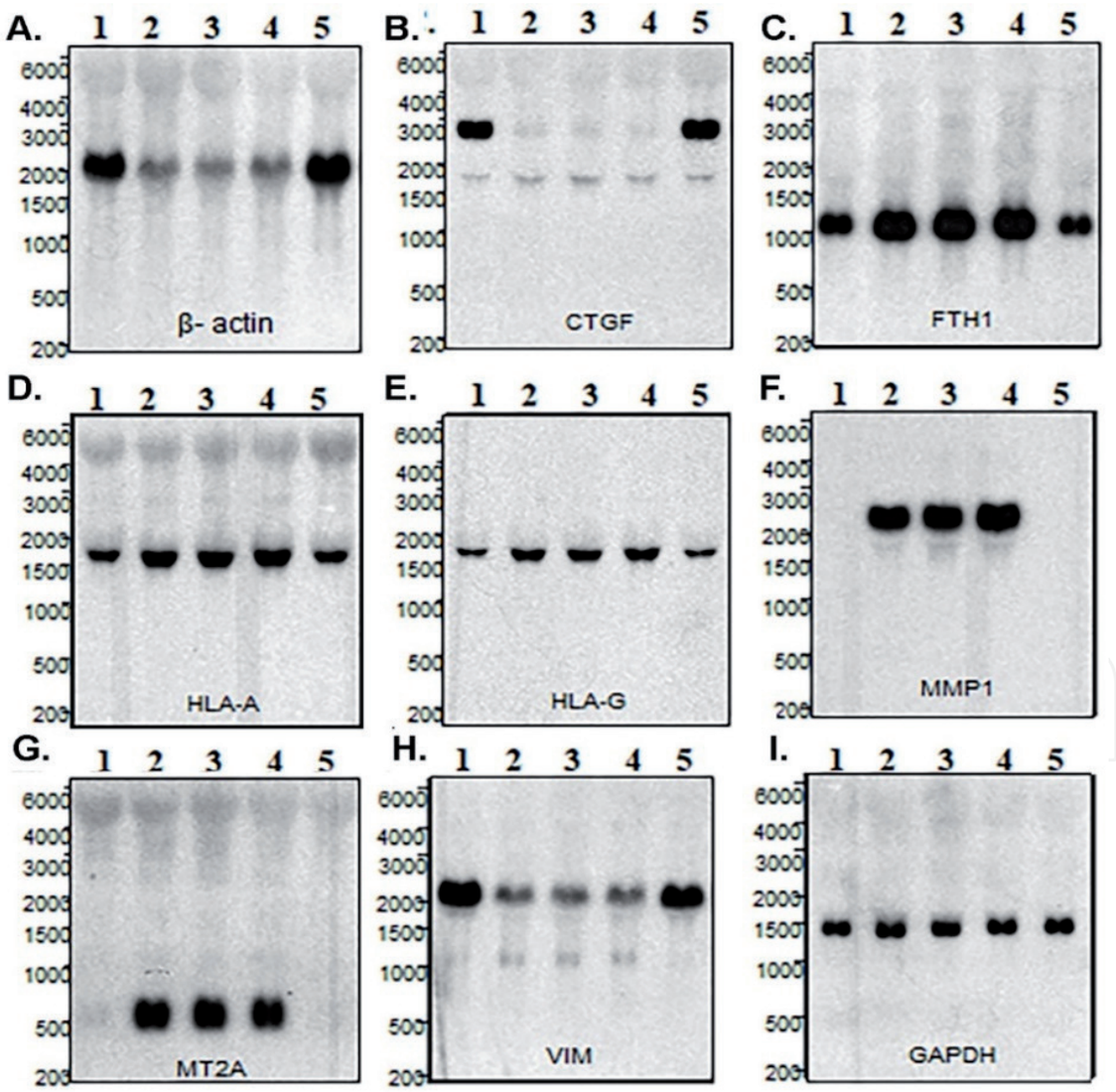

Figure 3.
Microarray data validation using northern blot analysis. 10 μg of total RNA of the fibroblast cells from each of the three RWV bioreactors (3-D spheres) were loaded in lanes 2–4. Lanes 1 and 5 are control RNA samples from the cells grown at normal gravity. The northern blot was probed and striped repeatedly using cDNA probe fragments from genes indicated at the bottom of each panel (A–I). RNA size markers in nucleotides were labeled along the left side of each panel.

that the mRNA levels of β-actin, CTGF, and VIM were down regulated, while that of FTH1, HLA-A, HLA-B (data not shown), HLA-G, MMP1, and MT2A were upregulated (**Figure 3**). In addition, Northern analysis showed that the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was consistent in the presence and absence of microgravity (**Figure 3I**).

3.4 Comparison of HDF in SMG gene expression data with that from the NASA twins study

The NASA Twins study is very substantial in undertaking with extensive data sets [14]. It was very appealing to compare the current data of HDF in SMG with the recently published gene expression data from the NASA Twins study. Since a large amount of data is available, it is more manageable to first focus on the gene expression data from the samples taken from inflight first half (up to 6 months) and the second half (up to 1 year), generated from the inflight astronaut's PBMCs. Such comparison of the two gene expression data sets gave 194 overlapping differentially expressed genes, or about 72%, of the genes found to be differentially regulated in the current study with HDF in SMG were also differentially regulated in the selected data set from the NASA study. Of these 194 genes, 86 had a similar expression pattern (regulated in the same direction) to the HDF in SMG data and 108 had the opposite expression pattern. A Heatmap was generated as a way of better visualizing the similarities between the data points (**Figure 4**).

3.5 KEGG pathways of the overlapping genes

As a way of comparing and better understanding the potential relationships between the HDF SMG gene expression data and the NASA Twin gene expression data from **Table 2** [16], we uploaded the gene list of the genes that showed same direction expression patterns (**Table 2**) and the genes that showed opposite expression patterns (**Table 3**) to DAVID in order to generate KEGG Pathway information.

After processing the list of genes with the same expression patterns through DAVID, a total of 7 KEGG pathways were statistically significant ($P \leq 0.05$) Interestingly, the genes enriched in all 9 KEGG pathways had >3.5 fold enrichment (**Table 2**). All the genes represented in the KEGG pathways were down-regulated in both sets of data. The Ribosome pathway had the greatest number of genes represented. The down-regulation of the ribosomal protein genes is consistent with our previous study in keratinocytes in SMG [21].

KEGG pathway analysis of the list of genes with opposite direction of expression regulation produced a total of 15 KEGG Pathways that were statistically significant ($P \leq 0.05$). Interestingly, the genes in all the 21 KEGG pathways had >2.5 fold enrichment (**Table 3**).

The protein processing in endoplasmic reticulum (ER) pathway had the greatest number of genes represented. In the current study of HDF in SMG, the genes represented in the ER pathway were all up-regulated whereas they were down-regulated in the NASA Twin Study data [16]. Heat shock proteins HSPA1A and HSPA8 have been identified as being gravity sensitive in several studies. In some of these studies, HSPA1A has been shown to be up-regulated [21, 32, 41] and in one study in addition to the NASA Twin Study was shown to be down-regulated [42]. Collagens also seem to be mixed in differential regulation in microgravity. In our current study, COL1, 2, and 6 were down-regulated but were up-regulated in the Twin study. In some studies COL1 has been shown to be up-regulated [43] while in others it has been shown to be down-regulated [33, 44]. FN1, which we identified as a putative

Figure 4.
Heatmap comparison of HDF in SMG gene expression data with that from the NASA twins study Heatmap is used to compare the HDF SMG data (column A) with the inflight, first half of the twins study ribodepleted (column B), PolyA+ (column C), multivariant (column D), and the inflight, second half of the twins study ribodepleted (column E), PolyA+ (column F), and multivariant (column G). Green were down-regulated genes, red represented up-regulated genes, and gray indicates the presence of no corresponding data.

Pathways	P Value	Gene name	FE
hsa03010:Ribosome	3.93×10^{-27}	RPL35, RPL27A, RPL36, RPS15A, RPS2, RPL39, RPS3, RPL32, RPL7, RPL31, RPS3A, RPL9, RPL34, RPL11, RPL10A, RPL12, RPS23, RPS24, RPL27, RPS7, RPS18, RPS19, RPL18A, RPS17, RPS10	22.18
hsa04530:Tight junction	5.32×10^{-3}	ACTB, ACTG1, MYL12B, MYL12A, MYL9	6.936
hsa05130:Pathogenic Escherichia coli infection	8.07×10^{-3}	ACTB, ACTG1, ARPC2, TUBA1B	9.465
hsa04670:Leukocyte transendothelial migration	1.40×10^{-2}	ACTB, ACTG1, MYL12B, MYL12A, MYL9	5.247
hsa04510:Focal adhesion	2.55×10^{-2}	ACTB, ACTG1, MYL12B, MYL12A, CAPN2, MYL9	3.515
hsa04810:Regulation of actin cytoskeleton	2.74×10^{-2}	ACTB, ACTG1, ARPC2, MYL12B, MYL12A, MYL9	3.448
hsa04921:Oxytocin signaling pathway	3.32×10^{-2}	ACTB, MYL6, ACTG1, CALM2, MYL9	4.023
hsa04611:Platelet activation	8.88×10^{-2}	ACTB, ACTG1, MYL12B, MYL12A	3.713
hsa05131:Shigellosis	9.53×10^{-2}	ACTB, ACTG1, ARPC2	5.657

The gene symbols in green color indicated down-regulation. The significance of gene enrichment is evaluated chiefly by the p value with a modified Fisher's exact test. FE is a measure of the magnitude of gene enrichment. P value ≤0.05 and FE ≥1.5 were considered significant and interesting, respectively.

Table 2.
KEGG pathways generated from comparison of genes differentially regulated in the same direction between the HDF and NASA twins data.

Pathways	P value	Gene name	FE
hsa04141:Protein processing in endoplasmic reticulum	2.93×10^{-5}	HSP90B1, VCP, DAD1, DNAJA1, PDIA6, HSPA1A, HSPA5, PDIA4, HSPA8, SSR1	5.81
hsa04512:ECM-receptor interaction	1.70×10^{-4}	CD44, COL3A1, COL6A3, COL1A2, COL1A1, ITGB1, FN1	7.91
hsa04612:Antigen processing and presentation	7.62×10^{-4}	HLA-A, HLA-C, HSPA1A, HLA-B, HSPA8, B2M	7.76
hsa04978:Mineral absorption	7.86×10^{-4}	ATP1B3, ATP1A1, MT1X, FTH1, FTL	11.2
hsa04974:Protein digestion and absorption	1.48×10^{-3}	ATP1B3, COL3A1, COL6A3, COL1A2, ATP1A1, COL1A1	6.7
hsa04918:Thyroid hormone synthesis	4.41×10^{-3}	HSP90B1, ATP1B3, ATP1A1, HSPA5, PDIA4	9.1
hsa04510:Focal adhesion	1.37×10^{-2}	CAV1, COL3A1, COL6A3, COL1A2, COL1A1, ITGB1, FN1	7.02
hsa05134:Legionellosis	1.48×10^{-2}	ARF1, VCP, HSPA1A, HSPA8	3.44
hsa04151:PI3K-Akt signaling pathway	1.67×10^{-2}	HSP90B1, COL3A1, COL6A3, COL1A2, YWHAQ, COL1A1, ITGB1, FN1, DDIT4	3.34
hsa05416:Viral myocarditis	1.71×10^{-2}	CAV1, HLA-A, HLA-C, HLA-B	6.9
hsa04144:Endocytosis	2.74×10^{-2}	CAV1, ARF1, HLA-A, HLA-C, HSPA1A, HLA-B, HSPA8	2.56

Pathways	P value	Gene name	FE
hsa05169:Epstein–Barr virus infection	2.92×10^{-2}	CD44, VIM, HLA-A, HLA-C, HLA-B	4.64
hsa05332:Graft-versus-host disease	3.96×10^{-2}	HLA-A, HLA-C, HLA-B	4.06
hsa05205:Proteoglycans in cancer	4.23×10^{-2}	CAV1, CD44, CD63, DDX5, ITGB1, FN1	2.85
hsa05330:Allograft rejection	4.87×10^{-2}	HLA-A, HLA-C, HLA-B	8.93
hsa04514:Cell adhesion molecules (CAMs)	5.37×10^{-2}	HLA-A, HLA-C, HLA-B, ITGB1	7.97
hsa04145:Phagosome	6.32×10^{-2}	HLA-A, HLA-C, HLA-B, ITGB1	3.46
hsa04940:Type I diabetes mellitus	6.60×10^{-2}	HLA-A, HLA-C, HLA-B	3.28
hsa03050:Proteasome	7.16×10^{-2}	PSMB4, PSMB7, PSMA6	7.02
hsa05320:Autoimmune thyroid disease	9.55×10^{-2}	HLA-A, HLA-C, HLA-B	6.7
hsa05145:Toxoplasmosis	9.79×10^{-2}	PPIF, HSPA1A, ITGB1, HSPA8	5.67

The green indicates genes that were down-regulated and the red indicates genes that were up regulated in the HDF data set. The significance of gene enrichment is evaluated chiefly by the p value with a modified Fisher's exact test. FE is a measure of the magnitude of gene enrichment. P value ≤0.05 and FE ≥1.5 were considered significant and interesting, respectively.

Table 3.
KEGG pathways generated from a comparison of genes differentially regulated in the opposite direction between the HDF and NASA twins data.

"space gene" [17, 18] has also been shown to have variations in expression patterns. It has been shown to be down-regulated in several studies [34, 44, 45] and up-regulated in others [16, 43, 46].

4. Discussion

In the current study, the gene expression profile of HDF grown in 5 day SMG was first displayed and validated (**Figures 1–3**, **Table 1**). The high throughput cDNA microarray data of HDF in 5 day SMG was then used to compare with the high throughput RNA sequencing data from an astronaut's PBMCs during a long term inflight ISS (**Figure 4**, **Tables 2** and **3**). Amazingly, about 72% of the 271 microgravity sensitive genes of HDF in SMG, were also differentially regulated in the NASA Twins 6- and 12-month inflight data. These 194 overlapping genes were identified as putative microgravity sensitive space genes, because the human dermal fibroblast cell line was only exposed to SMG, no radiation nor other space related environmental factor was involved. However, other factors such as cell type difference and space radiation, may also influence the expression of microgravity sensitive genes. Indeed, among these microgravity sensitive genes, 86 genes showed the same expression pattern in both simulated and real microgravity conditions while the other 108 genes displayed opposite direction of regulation.

It is remarkable that as many as 86 genes were found to have the same directions of expression regulation between very different settings of studies. It is most likely that these genes were the main players in cellular response to microgravity environment. When the 86 microgravity sensitive genes with the same expression regulation trends were subjected to KEGG pathway analysis, they were represented in seven significant pathways where they were all downregulated (**Table 2**). Most notably, both sets of global gene expression data showed the down regulation of 25 ribosomal protein genes. The genes in pathogenic Escherichia coli infection pathway and leukocyte trans-endothelial migration pathway were all down-regulated in

microgravity (**Table 2**), which may contribute to the decreased immune resistant to microbial infection in spaceflight. In addition, genes in the cytoskeleton network (ACTB, ACTG1, ARPC2, MYL12B, MYL12A, MYL9), focal adhesion (ACTB, ACTG1, MYL12B, MYL12A, CAPN2, MYL9), as well as tight junction (ACTB, ACTG1, MYL12B, MYL12A, MYL9) pathways were also downregulated in both sets of data. In combination with the downregulation of extracellular matrix proteins (**Table 1**) such as COL1A1, COL1A2, COL3A1 in HDF, the data indicated an overall decrease in bone matrix and skeletal muscle synthesis and increased catabolism (e.g. MMP1 increased sharply). Furthermore, genes in the oxytocin signaling pathway (ACTB, MYL6, ACTG1, CALM2, MYL9), which is involved in smooth muscle contraction and stress management, were also down in their expression levels in both microgravity data sets. Malfunction of this pathway has been implicated in depression, autism, and schizophrenia [47]. Overall, the data in **Table 2** gave a strong mechanistic connection to the main symptoms, such as skin problems and immunological stress, vascular changes, muscle atrophy and bone density alteration that were associated with the weightlessness of space flight. Indeed, the altered expression of these 86 microgravity sensitive genes affected many fundamental molecular functions (data not shown), including structural constituent of ribosome, RNA binding, protein binding, metal ion binding, structural constituent of the cytoskeleton, cadherin binding involved in cell-cell adhesion, extracellular matrix binding, etc. Many biological processes in these cells (data not shown), such as SRP-dependent co-translational protein targeting to membrane, translation initiation and elongation, mRNA stability, muscle contraction, regulation of cell shape, among others, were also significantly impacted. These genes with common trend of expression regulation in microgravity would most likely expand the list of putative major space genes and microgravity sensitive pathways [17, 18]. A substantial amount of information was derived from the current work which may necessitate more detailed analysis and discussion in future communications.

The number of overlapping microgravity sensitive genes was substantial considering the many differences between the two study settings. The result from this comparative analysis further validated the effectiveness of the bioreactors for SMG cell culture. The identification of 86 genes (**Table 2**) with the same direction of regulation in two different study settings is very substantial and unique. These genes should be considered best candidates for major microgravity sensitive genes because one of the two studies, the current study, only involves simulated microgravity, while the other study, the NASA Twins Study involves true spaceflight environment with microgravity and space radiation. This comparative analysis here enabled the differentiation of the microgravity effect alone on the differentially expressed genes from the human astronaut spaceflight gene expression data. The identification of the overlapping significant genes regulated in the opposite direction rendered important insight into human gene activity changes in very different study systems. The HDF cell line in SMG versus the human astronaut in ISS, adjusted their expressions toward adaptation to the simple SMG as well as the true space environment of both space microgravity and space radiation. The 108 microgravity sensitive genes with opposite directions of expression regulation could also be of major significance in the microgravity adaptation process. Compared to the single cell line in SMG alone for the HDF cells, the cell samples from the inflight astronaut was exposed to various other factors such as space radiation, in addition to the microgravity of the ISS. The more complex space environment may require the significant genes to modify their expression toward adaptation. Expression patterns in this group of genes could provide insight into our understanding regarding the interplay among different cellular gene functions in human adaptation to microgravity and space radiation (**Table 3**).

Our previous studies on gene expression in HEK cells grown in SMG has 43 genes overlapping with the HDF data in the current communication; of which, 23 genes were regulated in the same direction and 20 were regulated in the opposite direction [21]. These two different types of cells require different culture conditions and perform different roles in the skin. It is understandable that they have their characteristic expression profiles in response to the simulated microgravity environment. However, the number of overlapping genes were also substantial. In a previous review paper comparing various microarray based gene expression studies on microgravity effects, an initial list of 129 genes were identified as putative microgravity sensitive genes or major space genes [17]. In the current study, 12 out of the 194 genes that were significantly differentially regulated in both the HDF cells and the PBMCs, are also in the group of the putative major spaces genes [17], with 4 genes regulated in the same direction (MMP1, GPNMB, RPL10A, and ANXA2) and 8 genes (CAV1, CD44, CD59, CYR61, FN1, HSPA1A, MT1X, and PDIA4) changed their expression in the opposite directions in response to microgravity. Continued microgravity research in space and the readily controlled simulated microgravity bioreactors would provide valuable information toward the identification of major gravity sensitive genes, or simply, the major space genes. With more data available, the molecular and cellular mechanisms underlying the microgravity response could be better understood. Elucidation the molecular mechanism of human space adaptation response is an important aspect toward safer space experience and human health in general. It is evident that continued microgravity research is beneficial to healthy living in space and on earth.

5. Conclusion

The identification of 271 genes of HDF significantly differentially regulated by SMG provided a set of data for more detailed mechanistic studies; 72% of these microgravity sensitive genes were also reported in the high throughput gene expression data in the recent NASA Twins' Study.

The identification of the large number of overlapping genes between the HDF in SMG and astronaut's PBMCs indicates microgravity alone, without space radiation, was able to elicit an adaptive response involving a set of about 200 genes.

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Abbreviations/glossary

ACTB	actin beta
ACTG1	actin gamma 1
ANXA2	annexin A2
ATP1A1	ATPase Na+/K+ transporting subunit alpha 1
ATP1B3	ATPase Na+/K+ transporting subunit beta 3
ATP50	ATP synthase peripheral stalk subunit OSCP
ATP5E	ATP synthase F1 subunit epsilon
B2M	beta-2microglobulin
CALM2	calmodulin 2
CAPN2	calpain 2

CARMAweb	comprehensive R based microarray analysis web frontend
COL	collagen
COX	cyclooxygenase
CTGF	connective tissue growth factor
CTSL	cathepsin L
CYR61	cysteine rich angiogenic inducer 61
DAD	defender against cell death 1
DAVID	database for annotation, visualization and integrated discovery
DNAJA1	DnaJ homolog subfamily A member 1
FLNA	filamin A
FN1	fibronectin
FTH1	ferritin heavy chain 1
FTL	ferritin light chain
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GO	gene ontology
GPNMB	glycoprotein NMB
HARVs	high aspect ratio vessels
HDF	human dermal fibroblast
HLA	human leukocyte antigen
HSP	heat shock protein
ISS	international space station
ITGB1	integrin beta 1
KEGG	kyoto encyclopedia of genes and genomes
MMP1	matrix metallopeptidase 1
MRPS6	mitochondrial ribosomal protein 6
MT	metallothionein
MYL	myosin light chain
ND	NADH–ubiquinone oxidoreductase chain
NDUFB4	NADH:ubiquinone oxidoreductase subunit B4
PBMCs	peripheral blood mononuclear cells
PDIA	Protein disulfide isomerase family A member
PPIF	peptidylprolyl isomerase F
RAB13	member RAS oncogene family
RCCS	rotary cell culture system
RP	ribosomal protein
RWV	rotating wall vessel
SMG	simulated microgravity
SSR1	signal sequence receptor subunit 1
TNFRSF1A	tumor necrosis factor receptor superfamily member 1A
TPM2	tropomyosin beta chain
TUBA1B	tubulin alpha 1 B
TUBB6	tubulin beta 6 class V
UBB	ubiquitin B
UCHL1	ubiquitin C-terminal hydrolase L1
UQCR11	ubiquinol-cytochrome C reductase, complex III subunit XI
VCP	valosin containing protein
VEGFB	vascular endothelial growth factor B

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